Genetic Variants of the *DDR1* Gene Are Associated with Vitiligo in Two Independent Brazilian Population Samples

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Vitiligo is a chronic disease characterized by macules devoid of melanin and identifiable melanocytes. Adhesion of melanocytes to the basement membrane by integrin CCN3 is mediated through collagen IV receptor DDR1. We hypothesize that genetic variants of the *DDR1* gene are associated with the occurrence of vitiligo. To test this hypothesis, we genotyped 10 *DDR1* tag single-nucleotide polymorphisms (SNPs) in 212 trios composed of an affected child and both parents. Associated markers were then genotyped in 134 independent, unrelated individuals with vitiligo and 134 unrelated controls. Allele T of tag SNP rs4618569 was associated with an increased risk for vitiligo in the family trios ($P = 0.002$, odds ratio (OR) = 5.27; 95% confidence interval (CI) = 1.59–17.40), whereas allele C of tag SNP rs2267641 was associated with an increased risk for vitiligo in both family-based and case–control populations ($P = 0.01$, OR = 3.47; 95% CI = 1.22–9.17; $P = 0.04$, OR = 6.00; 95% CI = 1.73–52.33, respectively). The best evidence for association in the trios was obtained for a haplotype composed of risk alleles of markers rs4618569 and rs2267641 ($P = 0.0006$). There was an age-dependent enrichment of rs4618569 T allele and rs2267641 C allele in early-onset affected individuals. In conclusion, we propose *DDR1* as a susceptibility gene for vitiligo, possibly implicating a defective cell adhesion in vitiligo pathogenesis.

Journal of Investigative Dermatology (2010) **130,** 1813–1818; doi:10.1038/jid.2010.34; published online 25 February 2010

INTRODUCTION

Vitiligo is an acquired systemic, chronic disease with unpredictable clinical evolution, classically characterized by macules devoid of melanin pigment and identifiable melanocytes (Le Poole *et al.*, 1993). More recently, it was shown that melanocytes are not completely absent on the depigmented epidermis (Tobin *et al.*, 2000). Vitiligo can be classified into segmental or non-segmental, with different ethiopathological origins being proposed for these two clinical forms of disease (Hann and Lee, 1996). Non-segmental vitiligo includes the focal and generalized subgroups, the latter being further subdivided into vulgar, acrofacial, and universal (Huggins *et al.*, 2005).

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Abbreviations: DDR1, *discoidin domain receptor 1; KP, Köebner phenomenon; LD, linkage disequilibrium; SNP, single-nucleotide polymorphism*

Received 1 December 2009; revised 8 January 2010; accepted 12 January 2010; published online 25 February 2010

The observation that healthy skin from individuals affected by non-segmental vitiligo showed melanocyte detachment after experimentally controlled skin friction (Gauthier *et al.*, 2003a) led to a theory in which vitiligo is a primary melanocytorrhagic disorder with altered melanocyte responses to skin trauma, inducing melanocyte detachment and subsequent transepidermal loss. The authors speculate that an autoimmune phenomenon might have been triggered during melanocyte injury, caused by antigen release and recognition by dendritic cells or memory T cells, aggravating melanocyte detachment and loss (Gauthier *et al.*, 2003b).

Discoidin domain receptor 1 (DDR1) is a transmembrane tyrosine kinase receptor activated by several collagens (Vogel, 1999) that functions independently of β1 integrins (Shrivastava *et al.*, 1997). Collagen activation of DDR1 has impact over several functions of human leukocytes, such as cell differentiation, adhesion, and cytokine production (Yoshimura *et al.*, 2005). It is noted that DDR1 mediates E-cadherin (Wang *et al.*, 2009) and matricellular integrin CCN3-dependent cell adhesion to collagen type IV (Fukunaga-Kalabis *et al.*, 2006). Functional assays show inhibition of DDR1 by imatinib mesylate (Gleevec) (Day *et al.*, 2008), a potent inhibitor of BCR–ABL tyrosine kinase and c-Kit tyrosine kinase (Buchdunger *et al.*, 2000). Interestingly, the use of imatinib can lead to vitiligo-like lesions (Tsao *et al.*, 2003; Legros *et al.*, 2005; Brazzelli *et al.*, 2006), possibly due

to inhibition of tyrosinase activity through the c-kit pathway blockade (Cario-Andre *et al.*, 2006). The *DDR1* gene is located between *HLA-E* and *HLA-C* genes at chromosomal region 6p21 previously linked to vitiligo susceptibility in a Chinese population (Liang *et al.*, 2007).

Molecular genetic epidemiology tools have been used in linkage and association studies aiming to locate and identify the genes and the variants involved with vitiligo phenotypes. Here, we present data of a combined family-based and case–control association analysis between vitiligo and genetic variants of the gene *DDR1* involved in the regulation of melanocyte attachment to the base membrane of the epidermis. We selected 10 tag single-nucleotide polymorphisms (SNPs) that completely captured the information of the *DDR1* locus. These markers were genotyped in 596 individuals distributed in 212 family trios composed of one affected individual and both parents, ideal for genetic association analysis. Positive signals of association were then tested in an independent case–control population sample of 134 vitiligo-affected individuals and 134 unrelated controls, both groups recruited from the same geographic area in the south of Brazil as the family trios.

RESULTS

Our recruitment strategy led to the enrollment of 596 (225 affected) individuals distributed in 188 nuclear pedigrees from which 212 family trios could be derived. In addition to the family trios, we also enrolled a case–control population sample composed of 134 vitiligo patients and 134 control individuals fully matched by age, gender, and ethnicity. Table 1 summarizes and compares the characteristics of the affected individuals of the two studied populations. The median age of onset of the probands from the family-based sample was 18.7 ± 12.1 years, in contrast with 26.35 ± 17.01 in the case group of the case–control sample ($P = 0.0001$). A higher proportion of family history of vitiligo was observed in the family-based group, compared with the population-based sample ($P = 0.001$). Borderline difference in the distribution of the clinical form of disease, classified as segmental and non-segmental, was observed across the two samples ($P = 0.04$). There were no significant differences between the two population samples for distribution of gender and presence of autoimmune comorbidity. The most frequent autoimmune comorbidities observed in the family trios were autoimmune thyroid disease (15.3%), halo nevi (7.9%), and psoriasis (1.6%); among the cases of the case–control sample, the proportions were 15.6, 3, and 4.5%, respectively. Of the 212 probands of the family-based sample, 81.69% self-reported European ancestry and 15.96% reported a mixed Caucasian, African, and Native American background. African or Japanese ancestry was self-reported by two individuals each, and one individual reported an Iranian ethnic background. Affected individuals from the case–control population sample self-reported European (79.10%), mixed (19.40%), and African (1.50%) ancestry. The genotyping success rate was 95% or higher for all markers tested. Allele frequencies for all markers were in Hardy–Weinberg equilibrium in both population samples included in the study.

Table 1. Demographic and clinical characteristics of cases from both studied population samples

	Family-based *n*=225	Case–control *n*=134	*P*-value
Age of vitiligo onset (years)			
Mean (± SD)	18.7 ± 12.1	26.35 ± 17.01	0.0001[1]
Gender, n *(%)*[2]			
Male	97 (43.1)	55 (41)	0.701[3]
Female	128 (56.9)	79 (59)	
Autoimmune-comorbidity, n *(%)*[2]			
With	105 (46.7)	54 (40.3)	0.29[3]
Without	106 (47.1)	69 (51.5)	
Type, n *(%)*[2]			
Segmental	35 (15.5)	13 (9.7)	0.04[3]
Non-segmental	177 (78.7)	121 (90.3)	
Family history of vitiligo, n *(%)*[2]			
With	92 (40.9)	37 (27.6)	0.001[3]
Without	115 (51.1)	89 (66.4)	

[1] *t*-test.
[2] *n* = number of individuals with available information.
[3] χ^2 statistic.

Family-based association analysis

Family-based association analysis performed for all 10 SNPs detected significant association between vitiligo and six markers of the *DDR1 locus* that included the close neighboring gene *GTF2H4* (rs4618569, $P = 0.002$; rs1049622, $P = 0.01$; rs1049623, $P = 0.05$; rs226741, $P = 0.01$; rs1049628, $P = 0.01$ and rs2074510, $P = 0.04$). Out of these six associated SNPs, four were in strong linkage disequilibrium (LD) in our population sample and two (rs1049623 and rs2267641) were singletons. Odds ratio, as estimated by conditional logistic regression analysis, reached 5.27 for the marker rs4618569 (IC = 1.59–17.40) (Table 2). Haplotypic analysis involving the three independent tag SNPs associated with vitiligo in the family trios revealed strong evidence for association between vitiligo and haplotypes composed of risk alleles of markers rs4618569 and rs2267641 ($P = 0.0006$), rs1049623 and rs2267641 ($P = 0.008$), and all three tag SNPs ($P = 0.004$) (Table 3).

To further advance the understanding of the association effect observed, we performed a stratified analysis according to the non-genetic covariate age of diagnosis, presence of autoimmune comorbidity, and presence of family history of disease. Association signals for markers rs4618569 and rs2267641 were clearly concentrated among the trios with early age of onset of the affected child (Table 4), using both a cutoff of 18 and 25 years of age. For the other two covariates,

Table 2. Association between vitiligo and SNP markers of the *DDR1* locus in two independent population samples

	DDR1	*DDR1*	*DDR1*	*DDR1*	*DDR1*	*GTF2H4*
Marker name	**rs4618569**	**rs1049622**	**rs1049623**	**rs2267641**	**rs1049628**	**rs2074510**
Family-based analysis						
Bin structure[1]						
AF	0.77	0.77	0.62	0.77	0.77	0.78
Risk allele	T	C	T	C	G	G
FBAT (*P*-value)	0.002	0.01	0.05	0.01	0.01	0.04
OR (95% CI)	5.27 (1.59–17.40)	3.36 (1.15–9.75)	1.73 (0.97–3.08)	3.47 (1.22–9.17)	3.40 (1.17–9.80)	2.91 (0.99–8.48)
Case–control						
Risk allele	—	—	—	C	—	—
Univariate						
OR (95% CI)	NS	—	NS	6.00 (1.73–52.33)[2]	—	—
P-value				0.04		

Abbreviations: AF, allele frequency; CI, confidence interval; NS, not significant; *P*, reported for the best fit model; SNP, single-nucleotide polymorphism.
[1]A bin is composed of markers in strong linkage disequilibrium ($r^2 > 0.8$); any SNP in the bin is sufficient to explain the observed association with vitiligo.
[2]Case–control sample of 134 cases (genotype frequency CC/CT-99.2%) and 134 controls (genotype frequency CC/CT-95.1%).

Table 3. Family-based association analysis between vitiligo and two- and three-marker haplotypes

Haplotype						
rs4618569	**rs1049623**	**rs2267641**	**Haplotype frequency**	**Number of informative families**	**Z**	***P* (FBAT)**
T	—	C	0.72	35.9	3.4	0.0006
C	—	T	0.18	71.8	−0.3	0.74
T	—	T	0.05	20.2	4.5	0.54
T	T	—	0.61	59.6	2.2	0.02
C	T	—	0.22	74.3	−0.3	0.69
T	C	—	0.16	76.1	0.3	0.71
—	T	C	0.58	71.6	2.6	0.008
—	C	T	0.20	76.2	0.8	0.40
—	C	C	0.18	87.1	−1.5	0.13
T	T	C	0.58	63.7	2.8	0.004
C	C	T	0.18	66.5	15.8	0.47
T	C	C	0.14	67.9	−0.3	0.74

Only haplotypes with allelic frequency ≥ 0.05 were included and analyzed.

evidence for association did not resist stratification into each subgroup.

Case–control association analysis

To validate the association results described above, *DDR1* markers rs4618569, rs2267641, and rs1049623 associated with vitiligo in the family trios were genotyped and tested for association in an independent case–control population sample recruited from the same geographic area as the family trios. Linkage disequilibrium analyses confirmed independence of all three SNPs in the case–control sample, as observed for the family trios. Univariate analyses indicated statistically significant association between marker rs2267641 and vitiligo, with the same risk allele being observed for both populations ($P = 0.04$, OR = 6.00; 95% CI = 1.73–52.33) (Table 2; see Supplementary Table S1 online). As the controls were matched with the cases by gender, ethnicity, and age, no multivariate analysis was performed.

DISCUSSION

Genetic risk factors for complex traits have been intensively investigated over the past decade and candidate genes have been proposed for several common diseases, including vitiligo. Here, we present strong evidence for age-dependent association between vitiligo and variants of the *DDR1* gene in a large population sample of family trios from the south of

Table 4. Age-dependent genetic risk for vitiligo in the family trios

SNP ID	Risk allele	Age of onset (years)	*P*-value[1]	Odds ratio (95% CI)
rs4618569	T	⩽18	0.007	1.88 (1.18–3.01)
		>18	0.63	1.38 (0.36–5.30)
		⩽25	0.009	6.87 (1.6–29.18)
		>25	0.50	2.12 (0.23–18.83)
rs2267641	C	⩽18	0.03	1.64 (1.02–2.62)
		>18	0.10	1.85 (0.88–3.89)
		⩽25	0.03	3.76 (1.1–12.62)
		>25	0.35	2.66 (0.33–21.31)

Number of trios: 110 For ages ⩽18 and 89 for >18 years old; 143 for ages ⩽25 and 56 for >25 years old; 13 individuals, data not available.
[1]*P* reported for the best fit model.

Brazil. Family-based association and LD analysis of the *DDR1 locus* revealed three independent association signals corresponding to three tag SNPs, all intragenic to *DDR1*. When two- and three-marker haplotypes were constructed using the associated tag SNPs, association became more evident.

Interestingly, the association signal observed for two of the three independent disease markers was strongly dependent of age, using as a cutoff both the average age of diagnosis in our trios (18 years old) and the average age of diagnosis reported in the literature (Alkhateeb *et al.*, 2003) (25 years old, compatible with the average age of diagnosis in our case–control population sample). A similar age-dependent effect was recently observed for the *LTA* gene associated with leprosy in population samples of distinct ethnic backgrounds (Alcais *et al.*, 2007) and is in agreement with the hypothesis of a higher impact of genetic variants over populations with early onset complex diseases. It is noted that a recent, large association study on a Chinese population also revealed an influence of age over association between vitiligo and the promoter variant rs226977 of the gene *XBP1*, with borderline significance (Ren *et al.*, 2009).

No impact of the co-occurrence of autoimmune disorders was observed over *DDR1* association with vitiligo in our sample. Again, the same was observed in the *XBP1* study (Ren *et al.*, 2009), suggesting the existence of at least two groups of vitiligo susceptibility genes, as previously indicated by genome-wide linkage results (Spritz *et al.*, 2004): those acting over an autoimmune background, such as *NALP1* (Jin *et al.*, 2007), and others controlling additional, independent physiopathological mechanisms, such as *DDR1* and *XBP1*. Finally, both *DDR1* and *XBP1* (Ren *et al.*, 2009) associations are independent of the presence of family history of disease.

Genetic association studies involving a large number of tests are always prone to spurious positive findings because of multiple testing. To confirm association, all three *DDR1* tag SNPs associated with vitiligo in the family-based population were tested on an independent case–control sample, with positive evidence for association again being obtained for marker rs2267641 ($P=0.04$). It is possible that lack of association between the other two tag SNPs and vitiligo in the case–control sample is due to the age effect observed in the family trios—mean age of diagnosis of the cases of the case–control population was higher than 25 years, perhaps indicating a weaker genetic component controlling susceptibility to the disease as compared with the family-based sample. Nevertheless, perfect replication of the association signal for marker rs2267241, with the same risk allele on both population samples, argues strongly in favor of *DDR1* as a vitiligo susceptibility gene.

There are limitations in our study that should be addressed: (i) the loss of the association signal when the population sample of trios was stratified by the co-occurrence of autoimmune diseases and family history of vitiligo may be simply a consequence of loss of power due to a decrease in the sample size; (ii) the non-segmental clinical form of vitiligo is over-represented in both our samples—therefore, it is possible that *DDR1* is a gene associated exclusively with this particular form of disease. In fact, when segmental cases are removed from the family-based analysis, association persists for two out of three tag SNPs (rs4618569, $P=0.03$ and rs2267641, $P=0.02$) even with the reduction in the number of informative families; (iii) the small sample size of the case–control population certainly limits the power of the replication experiment. These first three limitations can be addressed by expanding the population samples. Finally, the MHC/HLA locus is classically known as presenting a very complex LD structure (Stenzel *et al.*, 2004) and it is possible that the *DDR1* alleles associated with vitiligo are in LD with the true causative variant located elsewhere within the region. Several HLA alleles have been associated with susceptibility to vitiligo in different populations (Zhang *et al.*, 2004; Yang *et al.*, 2005; Abanmi *et al.*, 2006). Further studies should address whether the association detected for *DDR1* markers is independent from other gene variants associated with vitiligo already described for this *locus*.

There is no obvious functional impact of the *DDR1* tag SNPs associated with vitiligo in this study. In addition, none of the known non-synonymous *DDR1* SNPs belong to any of the three associated bins described here. However, SNPs rs2267641 and rs1049623 are exonic, synonymous variants. Mechanisms by which synonymous SNPs could affect the protein function include impact over mRNA secondary structure with consequences on the level of protein expression (Nackley *et al.*, 2006). Furthermore, it has been recently shown that silent polymorphisms may deeply alter protein function – the authors propose a mechanism of modification of timing of co-translational folding and protein localization in specific cell compartments (Kimchi-Sarfaty *et al.*, 2007). How the associated variants of *DDR1* affect gene and, consequently, cell function remains to be investigated. One powerful strategy would be systematic comparative sequencing of the entire coding region of *DDR1* that will eventually lead to the identification of the true vitiligo causative variants. Further functional experiments involving tools such as gene expression and RNA stability analysis, gene silencing, and

immunohistochemistry would then be mandatory to establish the molecular defect responsible for the occurrence of the disease.

There is no consensus regarding exactly how melanocytes succumb to vitiligo (Schallreuter *et al.*, 2008). Here, we present *DDR1* as a susceptibility gene for vitiligo, highlighting a possible defective adhesion role in vitiligo pathogenesis: on the basis of our findings, one can speculate that vitiligo patients harboring *DDR1* variants have altered melanocyte adhesion to the basement membrane, particularly at trauma sites, contributing to local melanocyte disappearance.

MATERIALS AND METHODS

Population sample

This study was performed between December 2004 and November 2008. Affected individuals, their parents, and independent controls were enrolled from the Brazilian southern states of Paraná and Santa Catarina. Control individuals, matched with the cases by age, gender, and ethnicity were recruited from primary care centers located in the same geographical areas of the cases. Eligibility criteria for the control group were no self-reported history of autoimmune and inflammatory disease and no sign of vitiligo on complete physical examination by an experienced dermatologist. All patients and their relatives were ascertained at the outpatient vitiligo clinic of the Santa Casa de Misericórdia Hospital and a private medical office. On agreement to participate, the patient was asked to reach their parents and siblings to inform about the study and suggest voluntary contact with the investigators for possible enrollment and data collection.

All patients and relatives had their vitiligo status defined or confirmed by one single dermatologist (CCSC), who also performed disease classification of the confirmed cases. Diagnosis was performed on the basis of clinical findings after complete physical examination and Wood's lamp screening, and most of the individuals included were followed up during the period of the study. When necessary, confirmation of affected status was obtained on biopsy and histopathological examination.

Confirmed cases of vitiligo were classified as segmental and non-segmental disease, the latter including the focal, acrofacial, vulgar, and universal clinical forms. Patients were characterized as affected by segmental vitiligo if presenting unilateral or bilateral macules in a dermatomal or quasi-dermatomal distribution in one or more dermatomes. Focal vitiligo was characterized by one or more macules confined in a single specific area of the skin in a non-dermatomal distribution. Acrofacial vitiligo was characterized by macules in distal digits, and as periorificial on face and ano-genital areas. Minimal duration of acrofacial and focal vitiligo disease considering ''stable vitiligo'' was 1 year (Taieb and Picardo, 2007). Patients presenting lesions beyond acrofacial areas, such as the thorax, abdomen, thighs, legs, buttocks, arms, neck, and axillae, were classified as affected by vulgar vitiligo. Individuals affected by universal vitiligo presented few remaining normal macules of pigmentation on extended skin exam.

After diagnosis, all individuals with confirmed cases answered a questionnaire to determine gender, age of onset (self-reported as the date when the first white spot was observed), presence of family history of vitiligo as reported by the affected individual or a close relative, and occurrence of autoimmune diseases in the nuclear family, for example, among parents or siblings of the vitiligo-affected individual. Control individuals were asked about gender, age, and inflammatory and autoimmune diseases. All enrolled individuals had a blood sample collected and used for genomic DNA extraction following classic salting-out protocol (John *et al.*, 1991).

The study was approved by the Pontifical Catholic University of Paraná Ethics Committee and was conducted according to the principles of the Declaration of Helsinki. Written, informed patient consent was obtained for all individuals enrolled.

Marker selection and genotyping

Tag SNP markers capturing the entire information of the *DDR1 locus* were selected according to the information available at the International HapMap Project website, release 21. All selected markers presented a minor allele frequency of 0.2 or more in at least four populations: Caucasian, Japanese, Yoruba, and Chinese Han. The cutoff parameter to define LD between two markers was an $r^2 > 0.8$. Following these criteria, the following tag SNPs were included: rs4618569, rs1264323, rs1264320, rs2267641, rs1049622, rs1049623, rs1049628, rs1264319, rs2074510, and rs1264307, the last two markers spanning into the neighboring gene *GTF2H4.* SNPs were genotyped in one or both of the following platforms: (i) Applied Biosystems StepOnePlus or Applied Biosystems 7500 polarized fluorescence TaqMan assay (Lee *et al.*, 1993); (ii) SEQUENOM MassARRAY, which uses the iPLEX assay to incorporate mass-modified terminal nucleotides in the SBE step that are then detected by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (Griffin and Smith, 2000). Genotypes were automatically called using the appropriate software with subsequent manual checking of accuracy. Five SNPs (rs4618569, rs12644323, rs1049622, rs1264320, and rs2267641) were genotyped in duplicate in the family-based sample.

Statistical methods

Demographic and clinical variables of the cases from both the family-based and the case–control population samples were compared using χ^2 and the *t*-test, when indicated.

On genotyping, families presenting Mendelian inconsistencies were removed from the analysis. Hardy–Weinberg equilibrium and LD estimations were carried out using Haploview software (Barrett *et al.*, 2005). SNP bins were constructed using pairwise r^2 estimates obtained from parental data from the family trios and the control group of the case–control sample, and were defined as a not necessarily contiguous set of variants, in which at least one SNP has $r^2 > 0.8$ with all the other SNPs of the bin (Hinds *et al.*, 2005).

As the family-based sample consisted of trios with no missing parental information, a classic transmission disequilibrium test was performed for family-based association analysis, as implemented by FBAT software V. 2.0.2.c (Horvath *et al.*, 2001). FBAT was also used for family-based haplotypic association analysis, and *P*-values were adjusted using the Monte Carlo haplotype test using the *hbat* procedure. For estimation of OR, alleles for which positive evidence for association was detected were analyzed by conditional logistic regression analysis (Schaid and Rowland, 1998) using the PHREG procedure, as implemented in SAS software v.9.1. The population-based study was carried out using classical univariate techniques as implemented in SAS software v.9.1.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank all affected individuals, their families, and healthy volunteers for agreeing to participate in the study. We thank Dr Beno Paciornick, Head Director of Hormocentro Clinical Laboratory, for sample collection support. We also thank the Molecular Medicine Laboratories of Hoffmann La-Roche Ltd. (Basel, Switzerland): Klaus Lindpaintner, Hanno Langen, Angelique Augustin, Olívia Spleiss, and Thomas Schindler for valuable support. A special acknowledgment is addressed to Dr Erwin Schurr, Dr Alexandre Alcaïs, and Dr Aurelie Cobat for valuable suggestions during the development of the study and preparation of the article. R. I. Werneck was supported by CAPES/PDEE and CAPES/PROSUP scholarships. L. M. Nascimento was supported by a ''Marcelino Champagnat Award'' scholarship. This work received financial support from the Research Office of the Pontifical Catholic University of Paraná. G Walker and E Nogoceke are Hoffmann La-Roche Ltd employees. C. C. S. de Castro received a scholarship from the Roche Fellowship Program—Genomic Technologies Applied to the Investigation of Innovative Therapeutics.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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